A

ANTITUMOR RESEARCH PRODUCTS, INC.

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December 4, 1997

Commissioner
United States Department of Commerce
Patent and Trademark Office
Washington, D.C. 20231

Dear Sir:

Enclosed is a Patent Application entitled "The Use of the Activated N-Terminal Sixteen Amino Acid Peptide of the Antineoplastic Protein (ANUP) as a Pharmacological Active Anti-tumor Agent."

I am also enclosing the Declaration for Patent Application and the Verified Statement claiming small entity status.

Kindly bill me for the filing fee -- Small Entity Status.

Sincerely,

Nathan H. Sloane
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Applicant or Patentee: NATHAN H. SLOANE
Serial or Patent No.: _____
Filed or Issued: _____
Title:

Attorney's
Docket No.: _____

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) & 1.27(b))--INDEPENDENT INVENTOR

As a below named inventor, I hereby declare that I qualify as an independent inventor as defined in 37 CFR 1.9(c) for purposes of paying reduced fees to the Patent and Trademark Office regarding the invention entitled .
described in:

- ☒ the specification filed herewith.
- ☐ application serial number _____, filed _____.
- ☐ patent number _____, issued _____.

I have not assigned, granted, conveyed or licensed and am under no obligation under contract or law to assign, grant, convey or license, any rights in the invention to any person who would not qualify as an independent inventor under 37 CFR 1.9(c) if that person had made the invention, or to any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

Each person, concern or organization to which I have assigned, granted, conveyed, or licensed or am under an obligation under contract or law to assign, grant, convey, or license any rights in the invention is listed below:*

- ☐ No such person, concern, or organization
- ☒ Persons, concerns or organizations listed below*

* Note: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

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I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NATHAN SLOANE		
NAME OF INVENTOR	NAME OF INVENTOR	NAME OF INVENTOR
Nathan Sloane		
Signature of inventor	Signature of inventor	Signature of inventor
Date DEC 2 '97	Date	Date

OPM No 0651-0011 (12/31/95)

DECLARATION FOR PATENT APPLICATION

Docket No. _____

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled USE OF THE N-Terminal (ANUP) SIXTEEN AMINO ACID PEPTIDE of the specification of which The angiogenic Protein as a Pharmacologically active anti tumor agent
(check one) ☒ is attached hereto.

☐ was filed on _____
as Application Serial No. _____
and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, § 1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)			Priority Claimed
(Number)	(Country)	(Day/Month/Year Filed)	☒ Yes ☐ No
(Number)	(Country)	(Day/Month/Year Filed)	☒ Yes ☐ No
(Number)	(Country)	(Day/Month/Year Filed)	☒ Yes ☐ No

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application.

(Application Number)	(Filing Date)	(Status – patented, pending, abandoned)
(Application Number)	(Filing Date)	(Status – patented, pending, abandoned)

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole or first inventor (given name, family name) NATHAN HOWARD SLOANE
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Full name of second joint inventor, if any (given name, family name) _____
Second Inventor's signature _____ Date _____
Residence _____ Citizenship _____
Post Office Address _____

THE USE OF THE ACTIVATED N-TERMINAL

SIXTEEN AMINO ACID PEPTIDE OF THE ANTINEOPLASTIC PROTEIN (ANUP) AS A PHARMACOLOGICALLY ACTIVE ANTI-TUMOR AGENT

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References Cited

U.S. Patent Documents

4,359,415 11/1982 Sloane
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U.S. Application Number 08/641,905 05/02/96 Sloane

OTHER PUBLICATIONS

Sloane et al. Biochemical Journal (1986), 234, pp. 355-362.
Pottathil et al, Cancer Res. Therapy and Control (1990), 1, pp. 193-198.
Struve et al. Cancer Res. Therapy and Control (1990) 1: pp. 225-230
Ridge and Sloane, Cytokine (1996) 8 pp. 1-5
Sloane and Davis, Tumor Targeting (1996) 2 pp 322-326.

ABSTRACT

The 16 amino acid peptide representing the partial N-terminal sequence of the Antineoplastic Protein (ANUP) is a highly active pharmacologically antitumor agent. The 16 amino acid peptide is about 50% as active as antitumor agent compared to the antitumor activity as the protein (ANUP) per se when tested as a tumor killer agent (in vitro) utilizing the human breast tumor cell line (MDA 231). The protein (ANUP) in the purified state also shows regression of both HeLa (human cervical tumor all line) and KB (human laryngeal cell line) implanted in nude mice (Sloane, Davis Tumor Targeting (1996) 2, pp 322-326. The nonapeptide is about 10% as active compared to the antineoplastic protein (ANUP) in the human breast tumor cell line in vitro assay system. Both peptides, the 9 amino acid peptide and the 16 amino acid peptide require presence of the detergent sodium dodecyl sulfate to activate the peptides for full pharmacological antitumor activity.

The ANUP N-terminal 16 amino acid peptide contains the following sequence (as L-Amino Acids):

1.	Pyroglu	
2.	Leu	L
3.	Lys	K
4.	Cys	C
5.	Tyr	Y
6.	Thr	T
7.	Cys	C
8.	Lys	K
9.	Glu	E
10.	Pro	P
11.	Met	M
12.	Thr	T
13.	Ser	S
14.	Ala	A
15.	Ala	A
16.	Cys	C

The use of the N-terminal Sixteen Amino Acid Peptide as a Pharmacologically Active Anti-tumor Agent

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to the use of the 16 amino acid peptide

which represents the partial N-terminal amino acid sequence of the Antineoplastic Protein (ANUP) as a pharmacologically active antitumor agent. The peptide is about 50% as active as the protein per se but only about one-tenth of the weight of the peptide is equivalent in activity of the protein (ANUP) on a molar basis (ca 10^{-9}M).

SUMMARY OF THE INVENTION

The present invention describes the pharmacologically anti-tumor activity of the 16 amino acid peptide which represents the partial N-terminal amino acid sequence of the Antineoplastic Protein (ANUP).

The 16 amino acid peptide is approximately one-half as active as the protein on a molar basis utilizing the human breast tumor cell line (MDA 231). However, only about one-tenth of the weight of the peptide is required when compared to the amount of protein for equivalent activity against the human breast tumor cell line. Both the protein and the peptide exert their action by killing the tumor cells (apoptosis) since electron microscopy studies showed complete degradation of the cells (Struve et al. Cancer Res. Therapy and Control (1990) 1: pp 225-230.

DESCRIPTION OF THE PREFERRED EMBODIMENT

The 16 Amino Acid Peptide

The synthetic hexadeca peptide (16 L-amino acids) has the following sequence:

1.	Pyroglu		9.	Glu	E
2.	Leu	L	10.	Pro	P
3.	Lys	K	11.	Met	M
4.	Cys	S	12.	Thr	T
5.	Tyr	Y	13.	Ser	S
6.	Thr	T	14.	Ala	A
7.	Cys	C	15.	Ala	A
8.	Lys	K	16	Cys	C

The peptide was synthesized by Research Genetics Inc., Huntsville, AL 35801; the peptide was pure as shown by HPLC (high performance liquid chromatography) and the molecular weight was checked by mass spectrometry (MS).

The pharmacological anti-tumor activity of the 16 amino acid peptide (P_{16})

The antitumor activity of the peptide (P_{16}) was assayed against the human breast tumor cell line (MDA 231) and its activity was compared to the in vitro antitumor effect of the "pure" protein (ANUP).

The assay for the pharmacological antitumor activities were performed as follows utilizing 96 well plates --

20,300 - 30,000 human breast tumor cells in L-15 medium (200 ul) containing 2.5% fetal calf serum and 100 ug gestamycin per ml (complete medium) were incubated at 37° in air for 120 hours; after this incubation period 50 ul of serially diluted P_{16} and ANUP were added to each well. The serial dilutions were prepared as follows: 2 mg each (the P_{16} and ANUP) were dissolved in 2 ml of complete medium containing 0.5% sodium dodecyl sulfate (SDS). The solutions were diluted in complete medium containing 0.05% SDS to a concentration of 350 ug per ml.

Dilution plates were prepared as follows:

100 ul of complete medium were added to each well and 50 ul of diluted P_{16} and ANUP were added to each well in row A thus 1.3 dilution was accomplished; 50 ul were serially diluted in the 100 ul of medium in rows B through H. Thus the range of concentrations were from 6 ug to 2 mg when 50 ul each dilution series were added to 200 ul of the complete medium containing the MDA cells. The plates were incubated for an additional 96-120 hours. The medium was poured off and after a 90-minute incubation with 50 ul neutral red dye (0.5 ml neutral red (0.25% in 25% ethanol (0.6 ml) diluted 5.5 saline - 0.16 mm HCl) the cells were washed twice with PBS (phosphate buffer saline) at room temperature. The concentration of living cells (since only living cells absorb the dye) was determined after adding 100 ul lysing buffer (50% ethanol in 0.05 m NaH_2 PO_4) the concentration of neutral red released in each well was determined using a Dynetech plate reader set at 550 mm. A unit of activity was defined as the concentration of ANUP and P_{16} for 50% killing.

Under these assay conditions the 50% end points were as follows:

ANUP 0.1 ug/well = 1.25×10^{-8}M

P_{16} 0.0 ug/well = 2.2×10^{-8}M

Thus P_{16} is about 50% as active as ANUP on a molar basis; whereas on a weight basis only one tenth of the peptide weight is equal in activity 10 times the weight of the protein (ANUP).

In the absence of SDS neither the peptide nor the protein showed any antitumor activity. Thus the detergent is probably necessary to form the correct geometrical shape for activity as described by Sloane and Davis Tumor Targeting (1996) 2, 322-326. The data utilizing P_{16} as an antitumor agent against the human breast tumor cell line (MDA 231) are as follows:

	Fraction of the Activity relative to ANUP
P_{16} no SDS	± no Activity
P_{16} + 0.005% SDS	0.04
P_{16} + 0.02% SDS	0.50
P_{16} + 0.05% SDS	0.50

I Claim:

1. The use of the 16 L-amino acid peptide representing the partial N-terminal sequence of the antineoplastic protein (ANUP) as a pharmacologically antitumor agent which kills human tumor cells (using the human breast tumor cell line as a model).

2. The sequence of this peptide is: pyroglutamyl-leucinyl-lysinyl-cysteinyl-tyrosinyl-threoninyl-cysteinyl-lysinyl-glutamyl-prolinyl-methioninyl-threoninyl-serinyl-alaninyl-alaninyl-cysteine.

3. The use of the detergent sodium dodecyl sulfate to activate the 16 amino acid peptide to a form that kills human tumor cells using the human breast tumor cell line as an example.